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QUATERNARY AMMONIUM COMPOUNDS IN INTACT PLANTS AND CELL SUSPENSION CULTURES OF *ATRIPLEX SEMIBACCATA* AND *A. HALIMUS* DURING OSMOTIC STRESS

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Key Word Index—*Atriplex halimus*; *A. semibaccata*; Chenopodiaceae; cell suspension culture; osmotic stress; betaine; choline.

Abstract—*Atriplex halimus* and *A. semibaccata* plants and seeds were collected from their natural habitat in Egypt. Seeds of the plants were germinated in a low salt garden soil in Germany. A clear quantitative correlation between salt content of the soil of both biotopes and the amount of quaternary ammonium compounds in plants from both sites was not observed, indicating that the environmental sodium chloride concentration is not the only factor that influences the synthesis of quaternary ammonium compounds. Cell suspension cultures were raised from both plant species and the osmotic pressure of the culture medium increased by either sodium chloride or multiple amounts of macroelements. It was found that the cell suspension cultures are similar to the intact plants in that both exhibit enhanced growth under moderate sodium chloride concentration, do not accumulate proline in response to osmotic stress but do produce quaternary ammonium compounds. A positive correlation between salt concentration and accumulation of quaternary ammonium compounds was observed in the cultured cells.

INTRODUCTION

In contrast to glycophytes, halophytic plants have the ability to grow and tolerate high concentrations of salts. The adaptation of such plants to a saline habitat of low water potential and high salt concentration is always accompanied by accumulation of inorganic ions in the plant [1]. Plants have developed different ways to cope with high salt concentrations. Thus osmotolerance may be a matter of a discontinuous sequestration of salt within a plant in order to keep sensitive organs (such as young developing leaves) free from salt [2]. On the other hand plants may excrete salts as is the case with *Atriplex* species which have salt bladders [3].

Inorganic ions may also be stored in vacuoles. As a consequence non-toxic organic solutes accumulate in the cytoplasm. These solutes are called compatible solutes and may play the role of balancing cytoplasmic and vacuolar water potential [4]. The quaternary ammonium compound betaine (i.e. glycine betaine) has been suggested to be the major organic solute accumulating in the cytoplasm of plants [5] in the Chenopodiaceae [1]. Salt induced betaine accumulation correlates with increase in betaine-aldehyde dehydrogenase mRNA in *Spinacea oleracea*. This may require modulation of a betaine biosynthesis gene, which has been sequenced [6].

The balance of cytoplasmic and vacuolar water potential may not be the only or the main role of betaine in osmoregulation. Indeed betaine alleviates *in vitro* the deleterious effect of high salt concentrations on the activity of enzymes. Thus betaine may also protect enzymes [7, 8]. While the understanding of the molecular basis of osmotolerance in bacteria is advanced [9], the mechanisms of salt tolerance in higher plants are a matter of dispute [2]. One of the reasons is that higher plants are much more complex than bacteria. Plant cell suspension cultures are similar to a bacterial culture in that single cells or cell aggregates grow in a controlled environment.

In a few cases callus or cell suspension cultures have been employed to investigate osmotolerance in higher plants [e.g. 8]. One of these studies suggests that the mechanism of salt tolerance in higher plants may not only lie at the organismic level (*vide supra*) but also at the cellular level [10]. In order to investigate the cellular response of higher plants to salt stress, plant tissue cultures may, therefore, be suitable although data on this important point are insufficient [11]. We show that cell suspension cultures raised from *Atriplex halimus* and *A. semibaccata* plants respond to osmotic stress by accumulation of quaternary ammonium compounds.

RESULTS

We started our experiments on both *Atriplex halimus* and *A. semibaccata* by investigating plants after growth in their natural habitat near Amriya (Egypt). Seeds were also collected and plants raised in a completely different environment, viz. the garden of the Institute in Münster, Germany. Samples of these plants were also analysed and simultaneously cell suspension cultures were raised. The response of these cultures to salt stress was subsequently investigated.

Analyses of soil samples are listed in Table 1. The soil samples were collected from the natural biotope of both

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Table 1. Analysis of soil samples collected at Amriya (Egypt) and Münster (Germany)

	Location	
	Amriya	Münster
Na^+ (mg kg^{-1})	346.0	2.4
Cl^- (mg kg^{-1})	374.9	14.2
$CaCO_3$ (mg kg^{-1})	34.6	3.8
Total amount of salts (%, weight/weight)	1.70	0.08
pH	8.1	7.0
PO_4^{3-} (mg kg^{-1})	15.0	103.0
Ca^{2+} (mg kg^{-1})	27.0	69.0
Mg^{2+} (mg kg^{-1})	51.0	10.0
Cu^+ (mg kg^{-1})	0.2	1.2
Organic substances (%, w/w)	5.74	4.36

plants near Amriya along the Cairo-Alexandria desert road. The analytical data of these soil samples contrast with the data obtained from the garden soil of the institute in Münster: The desert soil has a relatively high concentration of $CaCO_3$ and a large amount of NaCl. While the amount of organic substances is quite similar in each case, the phosphate level of the garden soil exceeds the amount of phosphate detected in the desert soil.

Because the desert soil contains such a high amount of NaCl (which is not unexpected) the plants grown in the desert should contain more NaCl when compared to the plants raised in the garden at Münster which was found to be true for both plant species (Table 2). In each case the plants grown in Egypt contain high salt concentrations when compared to those plants grown in Germany. Although both *Atriplex semibaccata* and *A. halimus* grow in the same locations, the latter species accumulates more NaCl than the former. One may also expect desert grown plants to contain a higher amount of choline and betaine. Therefore, these were isolated from both plant species and identified by chromatography and spectroscopic methods (see Experimental). Subsequently a quantitative determination of betaine and choline was carried out using two methods, which gave identical results (Table 3). Choline is a minor component while betaine occurs at a much higher concentration. As expected, *Atriplex semibaccata* contains more betaine when grown in the desert.

The situation is different, however, with the *Atriplex halimus* plant. This plant contains more quaternary ammonium compounds when grown in the low salt garden soil. This result was unexpected and requires interpretation (*vide infra*). As a response to salt stress organic compounds other than quaternary ammonium compounds, viz. amides or amino acids and in particular proline may accumulate [1]. We have, therefore, also analysed the pool of free amino acids after extraction of plant material with water. The amounts of amino acids in extracts of *Atriplex semibaccata* plants collected from both locations are shown in Table 4. The total amounts of amino acids in plants grown in garden soil exceed the amounts of amino acids present in the desert plant. This is particularly true for arginine and glutamine.

In subsequent experiments we established cell cultures of both plants grown in Münster, the growth curves of both cultures are depicted in Fig. 1. Fourteen days after inoculation the medium was exhausted and the cells start to disintegrate, resulting in a decline of dry weight. The callus cultures derived from each plant species were placed on agar containing increasing concentrations of

Table 2. Analysis of Na^+ and Cl^- (mg kg^{-1}) content of *Atriplex semibaccata* and *A. halimus* plants grown either near Amriya (Egypt) or in Münster (Germany)

Plant location	Na^+	Cl^-
A. semibaccata (Amriya)	3.4	3.1
A. semibaccata (Münster)	1.48	1.27
A. halimus (Amriya)	5.7	6.7
A. halimus (Münster)	1.64	1.88

Composition of different soils is given in Table 1.

Table 3. Analysis of betaine and choline isolated from *Atriplex semibaccata* and *A. halimus* plants grown either near Amriya (Egypt) or Münster (Germany)

Plant location	Betaine (mg g^{-1} dry wt)	Choline (μg g^{-1} dry wt)
A. semibaccata (Amriya)	1.39	27.5
A. semibaccata (Münster)	0.14	38.0
A. halimus (Amriya)	5.82	347.5
A. halimus (Münster)	6.69	69.4

Table 4. Quantitative determination of amino acids extracted from *Atriplex semibaccata* plants grown in Egypt or Germany

Amino acid	Egypt	Germany
Asp	2.347	4.552
Thr	0.892	0.338
Ser	0.548	0.388
$AspNH_2$	p	p
Glu	p	p
$GluNH_2$	0.242	1.229
Pro	2.345	3.151
Gly	0.066	0.290
Ala	2.284	1.609
Val	1.347	0.386
Cys	—	—
Met	0.222	—
Ile	0.875	0.250
Leu	0.655	0.166
Tyr	0.377	0.053
Phe	0.634	0.367
Gaba	0.133	0.898
Lys	0.419	0.180
His	0.013	0.005
Arg	0.780	3.570
sum	14.179	17.433

Amino acids are given as μmol g^{-1} dry weight.
p = present but quantitative determination not possible due to overlap with the response of another amino acid.

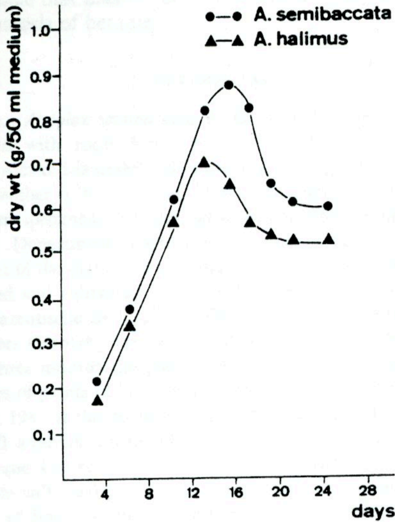

Fig. 1. Growth of cultured cells (250 ml medium) of *Atriplex semibaccata* and *A. halimus*.

macroelements [NaH_2PO_4, $CaCl_2$, $(NH_4)_2SO_4$, $MgSO_4$, KNO_3 and Fe(EDTA)]. In these media a 1.5-, 2-, 2.5-, 3-, 4- or 5-fold amount of macroelements was included. In parallel, agar media were prepared with increasing sodium chloride concentrations (0, 20, 60, 120 mM). Callus cultures were thus adapted to salt stress and the callus repeatedly transferred to new media containing the same type and amount of inorganic salts. From these salt-adapted calluses, cell suspension cultures were established from calluses of both *Atriplex* species. Thus, four different types of cultures were obtained: *Atriplex halimus* and *A. semibaccata* plant cells grown in the presence of increasing amounts of sodium chloride and macroelements.

Growth responses of the salt-adapted *Atriplex* cell cultures are shown in Fig. 2. The largest amount of tissue (fr. wt) was collected from the cultures containing 20 mM sodium chloride. Thus sodium chloride as opposed to macroelements at moderate concentrations stimulates growth of the cells. This conforms to observations with intact *Atriplex* plants [12].

When the four types of cultures were investigated for their ability to produce both betaine and choline (Figs 3 and 4) the following observations were made. While betaine exceeds the amount of choline present in intact plants significantly (Table 3) both choline and betaine are formed in both types (sodium chloride and macroelements) of cell cultures in similar amounts. In both types of cell cultures a direct correlation between salt concentration and quaternary ammonium compounds is seen and the amount of betaine isolated from the cell cultures is within the same order of magnitude when compared to the intact plants (Table 3) on a dry weight basis. Moreover, the accumulation of quaternary ammonium compounds is more pronounced in cultures with increasing concentrations of macroelements.

Proline was also determined in aqueous extracts of cells of the *Atriplex semibaccata* suspension cultures. A

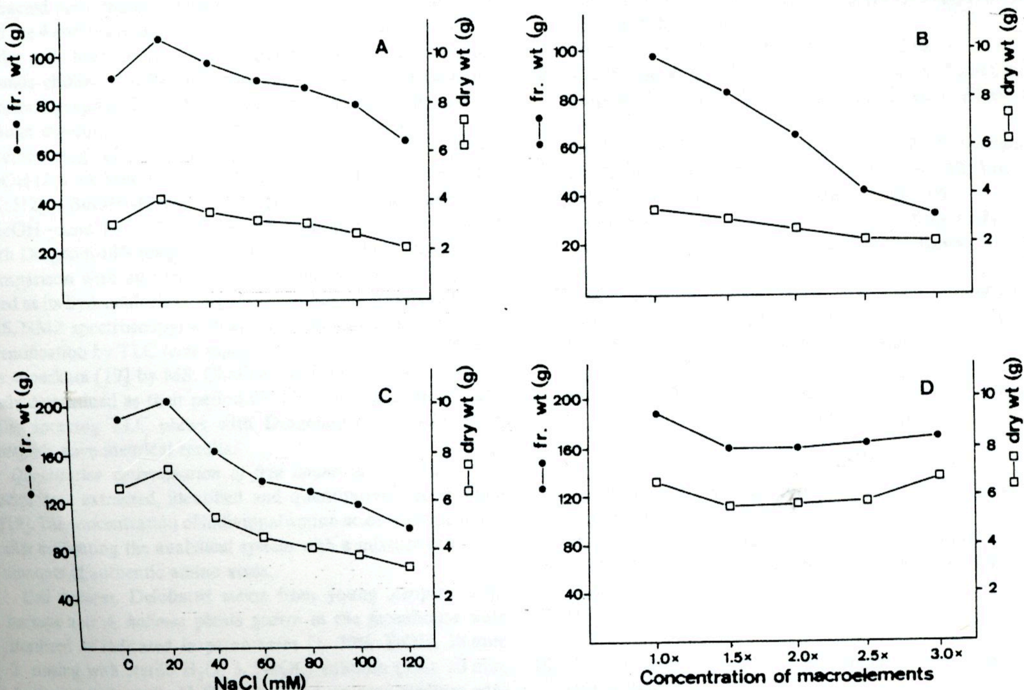

Fig. 2. Growth of cultured cells (250 ml medium) of *Atriplex halimus* (A, B) or *A. semibaccata* (C, D) incubated in the presence of increasing amounts of sodium chloride (A, C) or macroelements (B, D). The incubations were terminated 15 days after inoculation.

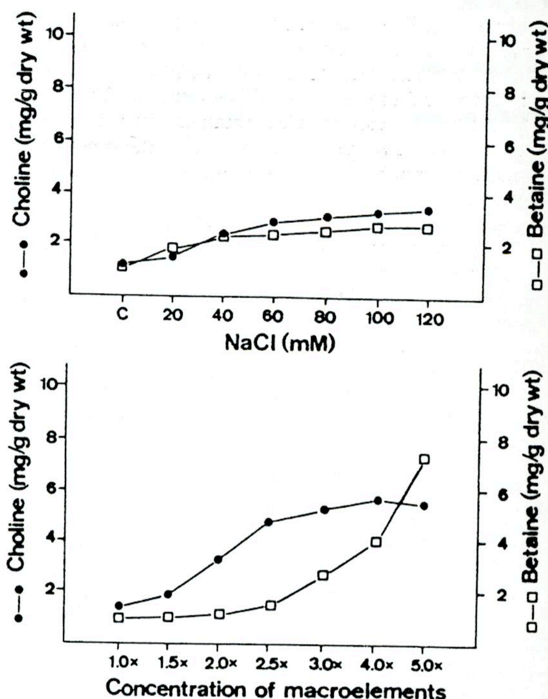

Fig. 3. Amounts of choline and betaine formed in the presence of increasing concentrations of sodium chloride or macroelements in *Atriplex halimus* cell suspension cultures.

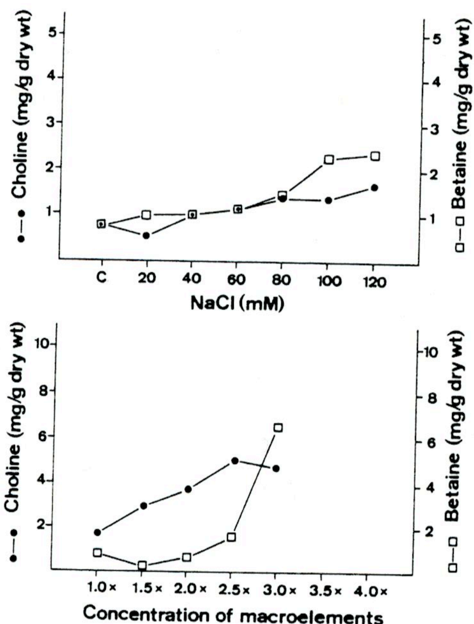

Fig. 4. Amounts of choline and betaine formed in the presence of increasing concentrations of sodium chloride or macroelements in *Atriplex semibaccata* cell suspension cultures.

direct correlation between concentration of sodium chloride or macroelements on one hand and proline on the other, was not observed; however, the proline extractable by water did not represent a relative large fraction of the total amino acids. The individual amino acids in extracts of the cells were also determined quantitatively. Their amounts varied considerably. There is only one amino acid which, as opposed to the intact plant (Table 4), occurred in a rather large amount (20- to 100-fold) in the cell suspension culture, viz. γ-aminobutyric acid (Gaba). The amount of γ-aminobutyric acid detected, however, did not correlate with the amount of salt to which cells were exposed.

DISCUSSION

When grown hydroponically under increasing external sodium chloride concentrations, *Atriplex* plants respond to environmental stress by accumulation of sodium chloride, betaine and proline [1]. This response, however, may not reflect the situation in the plant in its natural habitat because hydroponically grown plants live in an artificial environment without any limitations placed on growth by resource availability [2]. Observations with hydroponically grown plants may, therefore, be misleading when extrapolated for plants in their natural habitat. Indeed a clear quantitative correlation between salt and nitrogenous compounds was not seen when intact *Atriplex* plants were investigated after growth in their natural habitat (Tables 2 and 3). This indicates that there are also factors (such as age of plant, climate, water availability, day length) other than the environmental salt concentration that may influence the accumulation of quaternary ammonium compounds in *Atriplex* plants. Failure to observe betaine accumulation in *Atriplex* and *Salicornia* under osmotic stress has also been reported by other workers [13].

Hence attempts to explain susceptibility or resistance of plants to environmental stresses in terms of single factors are, therefore, unlikely to result in plausible theories of environmental adaptations. Changes in nitrogenous compounds can only be regarded as components of the resistance or tolerance mechanisms [2, 13]. Our observations, on the other hand, do show that accumulation of betaine is influenced by external salt concentrations. Indeed our experiments with cell suspension cultures show (cf. Figs 3 and 4) that single cells or aggregates of cells respond to osmotic stress by accumulation of choline and betaine. The cell culture system is similar to the intact plant in that both exhibit enhanced growth under moderate sodium chloride concentration, do not accumulate proline in response to osmotic stress under experimental conditions described herein, but do produce quaternary ammonium compounds.

Accumulation of quaternary ammonium compounds is not only triggered by sodium chloride but also by increased concentrations of macroelements, indicating that it is not sodium chloride *per se* that is responsible for their increased concentration in the cell culture but the osmotic stress mediated by increasing salt concentrations. We conclude again that stimuli other than high sodium chloride concentrations may contribute to the increased accumulation of nitrogenous compounds such as quaternary ammonium compounds.

The cell suspension cultures used in this study are heterotrophic cultures in which cells are grown on sucrose as an energy source. The biosynthesis of betaine from choline via betainal has been associated with chloroplasts of *Beta vulgaris* and *Spinacea oleracea* [28], plants which also belong to the Chenopodiaceae. Since

chloroplasts are not present in the *Atriplex* cell cultures it is possible that plastids in general are responsible for the biosynthesis of betaine.

EXPERIMENTAL

Plants. Atriplex semibaccata R. Br. and *A. halimus* L. were collected with roots from their natural habitat along the Cairo–Alexandria desert road near Amriya (Egypt) during June to September (1981–1983) at the flowering and fruiting stages. Voucher specimens are deposited in the Herbarium of the Botany Department, Faculty of Sciences, Cairo University. Samples of the plants were air-dried (30°). The plants were also collected and cultivated in the greenhouse of the Institute für Pharmazeutische Biologie und Phytochemie of the Universität Münster, Germany, during October to January 1982–1983. After three months the plants were transferred to the garden. Samples of plants of both species were collected during July to August 1983 at the flowering stage and also air-dried (30°).

NaCl analysis. Entire plants of the two species grown in Egypt and Germany were collected, and sand and externally adherent salt washed off with a small amount of cold water. Plants of both locations collected from both localities were treated identically, i.e. air-dried (30°), powdered and analysed for sodium chloride [15].

Soil analysis. Soil samples were collected from the natural habitat in Egypt and from the garden of the Institute. Each soil sample was collected from 10, 30 and 50 cm in an area of 4 m^2. The soil samples were analysed for inorganic ions, organic matter, pH and conductivity (total amount of salts) according to Than [16].

Isolation and identification of quaternary ammonium compounds. Dried and powdered plant material (100 g) was homogenized in boiling H_2O with an Ultra-Turrax mixer operated at 7000 rotations min^{-1} for 5 min and the suspension filtered. The extracted plant material was re-extracted ×3 with boiling H_2O and the 4 combined aq. extracts (*ca* 1 l) of each plant were concd under red. pres. to 20 ml. The quaternary ammonium compounds choline and betaine were isolated using ion exchange chromatography [17]. The fractions eluting from the ion exchange columns were tested for the presence of choline and betaine using TLC on silica gel 60 F_{254} HCO_2H–H_2O–*n*-BuOH (2:1:6); MeOH–H_2O (1:1); MeOH–Me_2CO–conc HCl (45:5:2); *n*-BuOH–H_2O–HCO_2H (12:7:1) or on alumina layer (MeOH–conc NH_3 (3:1). The chromatograms were sprayed with Dragendorff's reagent and choline and betaine detected by comparison with authentic material. Betaine was also crystallized as its hydrochloride [18] and identified by comparison (IR, MS, NMR spectroscopy) with authentic material. In addition to identification by TLC (*vide supra*), choline was also identified as its reineckate [19] by MS. Choline and betaine were quantitatively determined as their periodides [20] and densitometrically after spraying TLC plates with Dragendorff's reagent. Both methods gave identical results.

Quantitative determination of free amino acids. Free amino acids were extracted, identified and quantitatively determined [19]. The concentration of individual amino acids was calculated after calibrating the analytical system with a mixture of known amounts of authentic amino acids.

Cell cultures. Defoliated stems from young *Atriplex semibaccata* and *A. halimus* plants grown in the greenhouse were sterilized as indicated in parentheses [1. 70% EtOH, 18 min; 2. rinsing with sterile H_2O, 3. NaOCl solution (5%), 13 min; 4. rinsing with sterile H_2O] and placed on agar medium containing either Gamborg's B5 medium [22] (*A. semibaccata*) or medium A [23] (*A. halimus*). Calluses developed readily and were transferred to new media after 2 to 3 weeks. Callus tissue was then placed on solid media (0.8% agar) containing increasing concns of either NaCl or macroelements. The concns are given in the Figs and Tables. After several transfers on salt containing agar, cell suspension cultures containing identical salt concentrations were inoculated and after a growth period of several weeks transferred to new media containing again the same salt concentrations. Thus cells were gradually adapted to and then maintained either in or on salt containing media.

For determination of either proline or quaternary ammonium compounds the cells from 250 ml medium were collected by suction on a Buchner funnel and weighed. The fresh cells were then extracted with hot H_2O as described before and the quaternary ammonium compounds and proline were determined quantitatively as described [21, 22].

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ENANTIO- AND DIASTEREOSELECTIVITY IN THE BIOTRANSFORMATION OF CARVEOLS BY *EUGLENA GRACILIS* Z

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Key Word Index—*Euglena gracilis* Z; biotransformations; monoterpenoids; terpene alcohols; (−)-, (+)- and (±)-*trans*-carveols; (−)-, (+)- and (±)-*cis*-carveols; enantioselectivity; diastereoselectivity.

Abstract—The enantio- and diastereoselective biotransformation of (+)-, (−)- and (±)-*trans*-carveol and *cis*-carveol by *Euglena gracilis* Z cultured photoheterotrophically has been investigated. Though (−)-*cis*- and (+)-*trans*-carveols were not transformed at all, both (−)-*trans*- and (+)-*cis*-carveols were dehydrogenated to the corresponding carvones, which were further transformed *via* dihydrocarvones to dihydrocarveols. Furthermore, enantio- and diastereoselectivity in the biotransformation of (±)-*trans*- and (±)-*cis*-carveols and the mixture of diastereomers were observed.

INTRODUCTION

In previous papers [1, 2], we reported on the biotransformation of terpene and related aldehydes to the corresponding primary alcohols by *Euglena gracilis* Z cultured photoheterotrophically. In our continuing biotechnological studies of the metabolism of terpenoids by *Euglena*, we chose carveols (microbial and plant cell metabolites of *d*-limonene [3–5] and carvone [6]) as substrates with a view to developing a procedure for the useful utilization of *Citrus* oil as biomass. *Euglena* converted carveols with high enantio- and diastereoselective dehydrogenation to give carvone.

We now report on the enantio- and diastereoselectivity in the biotransformation of (−)-*trans*- and (−)-*cis*-carveol (**1** and **2**), (+)-*trans*- and (+)-*cis*-carveol (**6** and **7**), (±)-*trans*-carveol (**1** and **6**) and (±)-*cis*-carveol (**2** and **7**) by *E. gracilis* Z.

RESULTS AND DISCUSSION

The time courses for the biotransformations of compounds **1**, **2**, **6**, **7**, **1** and **6** and **2** and **7** by *E. gracilis* Z are shown in Fig. 1. Compound **1** was transformed stereospecifically via (−)-carvone (**3**) and (+)-dihydrocarvone (**4**) to (+)-neodihydrocarveol (**5**) as the major product (Fig. 2). Furthermore, when either **3** or **4** was used as the substrate, **5** was specifically formed. However, **2** was not transformed. On the other hand, in the cases of **6** and **7**, **7** was transformed via (+)-carvone (**8**) and (−)-isodihydrocarvone (**9**) to (−)-isodihydrocarveol (**10**) as major product and (−)-neoisodihydrocarveol (**11**) as minor product (Figs 1 and 2), whereas **6** was not transformed. Compound **8** was also transformed via **9** to **10** and **11**. When the mixture of acetates (**12** and **13**, 49:51, peak area in GC) was added into the culture broth, hydrolysis of both acetates occurred to give **6** and **7** (49:51, peak area in GC), of which only **7** was diastereoselectively transformed in the same manner as

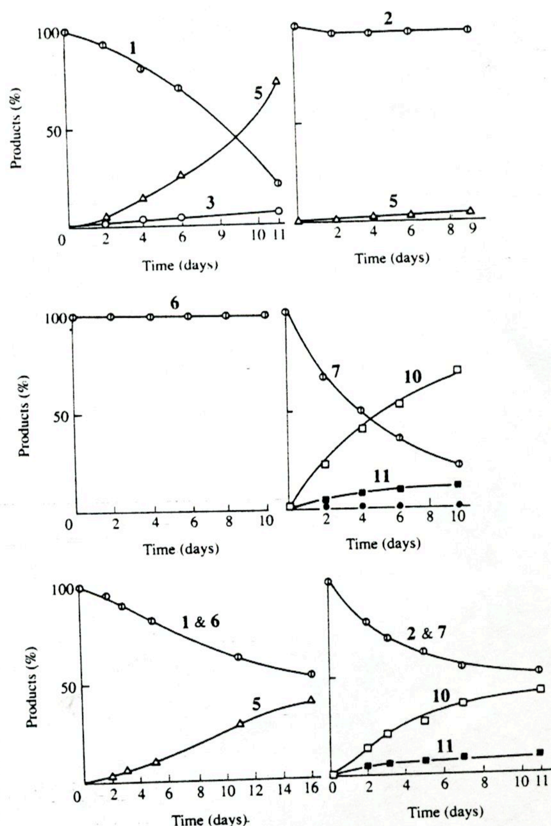

Fig. 1. Time courses for the biotransformations of (−)-*trans*- and *cis*-carveol (**1** and **2**), (+)-*trans*- and *cis*-carveol (**6** and **7**) and (±)-*trans*- and *cis*-carveol (**1** and **6**, and **2** and **7**) by *E gracilis* Z. **3**, (−)-carvone; **5**, (+)-neodihydrocarveol; **10**, (−)-isodihydrocarveol; **11**, (−)-neoisodihydrocarveol.